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International application number: PCT/GB05/000052

International filing date: 10 January 2005 (10.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: GB
Number: 0401304.1
Filing date: 21 January 2004 (21.01.2004)

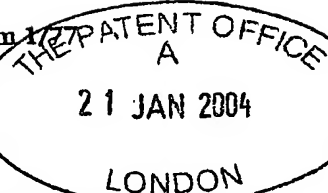Date of receipt at the International Bureau: 17 February 2005 (17.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

    0879 2400 001
    Patents ADP number *(if you know it)*

    DYNAMETRIX LIMITED
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    If the applicant is a corporate body, give the country/state of its incorporation

    UK

4. Title of the invention

    GENOTYPING METHOD

5. Name of your agent *(if you have one)*

    "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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    Patents ADP number *(if you know it)*   109006

	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
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   Description   24

   Claim(s)   4   *CF*

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11. I/We request the grant of a patent on the basis of this application.

    Signature(s)   *Heuler Ellis*   Date 21 January 2004

12. Name, daytime telephone number and
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population of melt curves,

wherein each of the grouping lines intersects a number of melt curves within said population,

(c) assigning genotype categories to said grouping lines, and;

5   (d) determining the genotype category of a nucleic acid sample in said population by identifying one or more grouping lines which intersect the melt curve of the sample.

10   The method may include an initial step of obtaining the melt curve data from a population of nucleic acid samples. Melt curves may be obtained for a population of nucleic acid samples by;

(a) contacting a population of nucleic acid samples
15   with one or more nucleic acid probes which hybridize with each of the samples to form a population of complexes,

(b) progressively altering the hybridization conditions to decrease or increase the formation of said complexes;

20   (c) measuring output signals indicative of the extent of hybridization of the complexes; and,

(d) plotting the output signals relative to the hybridization conditions for each of said population of complexes to produce a population of melt curves.

25

A melt curve may be a melting curve obtained by increasing the stringency of the hybridization conditions and monitoring the dissociation of the sample and the probe nucleic acids or an annealing curve obtained by
30   reducing the stringency of the conditions and monitoring the association of the sample and the probe nucleic acids.

methylguanosine, methylinosine, D-mannosylqueosine,

wybutoxosine, pseudouridine, queosine, thiocytidine and
thiouridine.

A nucleic acid strand may also comprise one or more
modifiers of base-pair stability.

5

The nucleic acid sample may be obtained by amplifying a
region of sample DNA containing one or more positions or
sites of variation. A single strand of the amplified
product may then be isolated and/or purified.

10

A position of variation is a position within the tested
sequence which may differ between samples obtained from
different individuals or between cells within an
individual, for example due to allelic variation,

15 polymorphism or mutation. For example, there may be an
insertion, deletion or substitution of one or more
nucleotides at a position of variation, relative to an
allelic reference sequence. In some embodiments, the
sample may contain a polymorphism at a position of

20 variation, for example a single nucleotide polymorphism.

Sample nucleic acid from an individual may be subjected
to a specific amplification reaction such as the
polymerase chain reaction (PCR) (reviewed for instance in

25 "PCR protocols; A Guide to Methods and Applications",
Eds. Innis et al, 1990, Academic Press, New York, Mullis
et al, Cold Spring Harbor Symp. Quant. Biol., 51:263,
(1987), Ehrlich (ed), PCR technology, Stockton Press, NY,
1989, and Ehrlich et al, Science, 252:1643-1650, (1991))

30 to generate the nucleic acid sample. DNA amplification
using techniques such as PCR are well-known in the art.

The nucleic acid probe may comprise or consist of the
nucleotide sequence of the most common allele of the

luminescent (fluorescence/phosphorescence) intensity or

decay time measurements, light polarization measurements, light absorption/transmission and reflectance measurements, chemiluminescence signals, scattered light patterns and evanescent fields. Methods for the

5   production and measurement of these output signals are well-known in the art.

A melt curve for a hybridization complex may be obtained by any convenient method. Many such methods are known in

10   the art.

A melt curve may consist of a graphic plot or display of the variation of the output signal with the parameter of hybridization stringency. Output signal may be plotted

15   directly against the hybridization parameter. Typically, a melt curve will have the output signal, for example fluorescence, which indicates the degree of duplex structure (i.e. the extent of hybridization), plotted on the Y-axis and the hybridization parameter on the X axis.

20   In other embodiments, melt curves may be provided by plotting the first or the second derivative of the output signal (or the negative values thereof) against the hybridization parameter.

25   Preferred hybridization parameters include temperature (or time, if temperature was altered steadily), pH and voltage.

In preferred embodiments, the population of melt curves

30   is normalised to a common start point prior to applying said grouping lines.

The population of melt curves may comprise one or more reference melt curves. Reference melt curves may be

In other words, grouping lines may be used repetitively

for a particular system once they have been established

for that system. There is therefore no requirement for
each melt curve population to comprise reference melt
curves.

5   The grouping lines may be applied to the population of
melt curves by an operator in order to categorise the
samples. In preferred embodiments, the population of
melt curves is provided by a data processing means and
displayed on a monitor or other image display. The
10   operator may then apply the grouping lines to the
displayed images manually by means of a graphic
interface. For example, the operator may apply grouping
lines to the melt curves displayed on the monitor using a
keypad, mouse, touchpad, trackball, pressure-sensitive
15   stylus, or other interface device. Suitable graphic
interfaces and interface devices are well-known in the art.

In other embodiments, the grouping lines may be applied
to population of melt curves automatically by a data
20   processor. Many different strategies for applying the
grouping lines are possible and can be readily
implemented by those skilled in the art. For example, the
data processor may be adapted to apply grouping lines by;
(i) tracking the Y-value distribution of said melt curves
25   along the X-axis, (ii) identifying one or more regions in
which said melt curves separate into distinct clusters,
and (iii) applying one or more grouping lines to define
each said cluster.

30   Alternatively, the data processor may be adapted to apply
grouping lines by; (i) applying a plurality of candidate
lines to the population of melt curves, and (ii)

A nucleic acid sample from a polyploid genome may have
more than three genotypes at a position of variation. In
addition to being homozygous for a match or a mismatch
with a reference allelic sequence, the sample may have
5 several heterozygous genotypes, depending on the number
of match and mismatch alleles present in the genome.
Further genotype categories are also possible if the
sample contains more than one position of variation or is
present in multiple copies in the genome.
10

As described above, each grouping line or combination of
grouping lines may be assigned to a genotype category
i.e. grouping lines are applied to the distinct clusters
in the population of curves and then assigned to each
15 genotype category. In other embodiments, grouping lines
preassigned to each genotype category may be applied to
distinct melt curve clusters in the population of curves.
For example, a grouping line may be applied to intersect
a cluster of curves that comprises a reference curve of a
20 known genotype.

Grouping lines may be assigned to a genotype category by
determining the context of the melt curves intersected by
the line within the population of curves. For example, a
25 grouping line which intersects melt curves that show only
a high temperature of melting relative to the population
as a whole may be assigned to samples which are
homozygotes for sequences that matched the probe. A
grouping line which intersects melt curves that show only
30 a low temperature of melting relative to the population
as a whole may be assigned to samples which are
homozygotes for sequences that mismatched the probe. A
grouping line which intersects melt curves that show both

A method may comprise;

    (a) providing reference melt curves for a population of nucleic acid molecules of one or more known genotype categories hybridized to a nucleic acid probe;

5   (b) applying one or more grouping lines to the population of reference melt curves,

    wherein each of the grouping lines intersects a one or more reference melt curves within said population,

    (c) determining the intersection of the grouping
10 lines by reference melt curves of each of the genotype categories; and,

    d) providing an assignment algorithm which relates each genotype category to the intersected grouping lines.

15 The genotype category of a nucleic acid sample in a population may then be determined by applying the one or more grouping lines previously applied to the reference curves as described above to a population of sample melt curves, identifying which of the one or more grouping
20 lines intersect the melt curve of the nucleic acid sample, and applying said assignment algorithm.

For example, a sample melt curve in an assay may intersect two or more grouping lines. The curve may be
25 assigned to a genotype category by the implementation of an algorithm that relates the grouping lines intersected by the curve to a genotype category. Use of such an algorithm obviates the need for reference curves in every population of melt curves.

30

In some embodiments, an assignment system or algorithm may consist of allocating an order of precedence to the grouping lines. The sample curve is then genotyped

an output signal indicative of the association or

dissociation of two or more components of a biological complex in response to a changing physical parameter, such as temperature. Such a dataset may be plotted as a melt curve.

5

Melt curves may be obtained for a population of biological complexes by;

(a) contacting, in a medium, biological components which bind together to form a population of biological

10   complexes,

(b) progressively altering the conditions of the medium to decrease or increase the formation of said complexes;

(c) measuring output signals indicative of the

15   extent of formation of the complexes; and,

(d) plotting the output signals relative to the conditions for each of said population of complexes to produce a population of melt curves.

20   Suitable biological components include biological molecules such as polypeptides, nucleic acids, lipids and carbohydrates and biological particles such as microbial or eukaryotic cells and viral particles.

25   A biological complex may, for example, include an antibody/antigen complex, a multi-component protein or protein complex, a receptor/ligand complex, an enzyme/inhibitor complex or a double stranded nucleic acid complex.

30

Suitable conditions that may be altered include pH, temperature and electrical field strength.

The term "computer program product" includes any computer readable medium or media which can be read and accessed directly by a computer. Typical media include, but are not limited to: magnetic storage media such as floppy
5 discs, hard disc storage medium and magnetic tape; optical storage media such as optical discs or CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media.
10

A typical computer system of the present invention comprises a central processing unit (CPU), input means, output means and data storage means (such as RAM). A monitor or other image display is preferably provided.
15 For example, a computer system may comprise a processor adapted to perform a method of the invention. For example the processor may be adapted;

(a) to produce a graphic plot for each of the datasets in a population to produce a population of
20 graphic plots,

(b) to display said population of plots

(c) to apply or to allow the user to apply one or more grouping lines to the displayed population of graphic plots,
25 wherein each of the grouping lines intersects one or more plots within said population,

(d) to assign each said grouping line to a category, and;

(e) to determine the category of one or more of the
30 datasets in said population by determining the grouping line which intersects the graphic plot of the one or more datasets.

In other embodiments, the processor may adapted to apply grouping lines by

(i) tracking the Y-value distribution of said plots along the X-axis,

5   (ii) identifying one or more regions in which said plots separate into distinct clusters; and,

(iii) applying one or more grouping lines to define each said cluster.

10   In other embodiments, the processor may be adapted to apply grouping lines by;

(i) applying a plurality of candidate lines to said population of plots, and;

(ii) identifying one or more candidate lines which only
15   intersect a discrete cluster of curves within said population as grouping lines.

In other embodiments, the processor may store the positions of grouping lines which have been assigned to
20   genotype categories on the basis of a population of reference curves and apply grouping lines at the stored positions on a population of sample curves. Thus, the processor may be adapted to apply grouping lines by;

(i) retrieving the stored positions of one or more
25   established grouping lines;

(ii) applying said established grouping lines to a displayed population of curves.

Annealing/denaturing data from a population of nucleic
30   acid samples hybridized to a nucleic acid probe may include the amount of an output signal at one or more different proteins and protein complexes, nucleic acid materials, acceptors and receptors, peptides, lectins,

The DNA hybridization device may further comprise a
hybridization chamber suitable for annealing or
denaturing nucleic acid complexes in accordance with the
invention. The device may comprise means for
5   progressively altering the hybridization conditions
within the chamber, for example by altering the
temperature, pH or voltage.

Various further aspects and embodiments of the present
10   invention will be apparent to those skilled in the art in
view of the present disclosure. All documents mentioned
in this specification are incorporated herein by
reference in their entirety.

15   Certain aspects and embodiments of the invention will now
be illustrated by way of example and with reference to
the figures described below.

Figure 1 shows DNA melt curves of primary non-normalised
20   data with applied grouping lines. Solid lines show
homozygous matches to the probe, thick-hatched lines show
homozygous mismatches and thin-hatched lines show
heterozygotes.

25   Figure 2 shows DNA melt curves of primary normalised data
with applied grouping lines.

Figure 3 shows DNA melt curves of negative first
derivative non-normalised data with applied grouping
30   lines.

Figure 4 shows DNA melt curves of negative first
derivative normalised data with applied grouping lines.

by 35 cycles of 94°C for 15 seconds and of 55°C for 30s.

Genotyping Assay

To create melt curves we employed the Dynamic Allele-Specific Hybridization (DASH) genotyping method (Genome
5 Research, 2003, 13:916-924). For this, a streptavidin
coated nylon membrane was pre-wet in HE buffer (0.05M
Hepes, 0.005M EDTA, pH 7.9) and clamped onto an opened
PCR plate (post-PCR) enabling the PCR products to be
centrifugally transferred onto the membrane
10 (Biotechniques, 2002, 32:1322-1329) to bind and create a
macro-array. The membrane was then rinsed in HE buffer
and immersed in 0.1M NaOH for 2 minutes to denature the
DNA and so remove the non-biotinylated PCR product
strand. A further rinse in HE buffer was used to
15 neutralize the pH of the membrane. Probe rs1133104+01P
(3'-ROX labeled oligonucleotide 5'-ttctctccCtgtgtgca-3',
with the captalized 'C' being the allele-specific base)
was then used at 2 pmol/ml in HE buffer to coat the
membrane. To drive probe annealing to completion the
20 membrane was placed between glass sheets, heated to 85°C,
and allowed to cool to room temperature. The probe was
thus annealed to the bound single strand of the PCR
product. A final rinse in HE buffer was used to remove
excess unbound probe.
25

To enable assessment of the degree of hybridization
between probe and PCR product at any point in time, the
membrane was soaked for 3 hours in 40ml HE-buffer
containing SYBR Green I dye at 1:20 000 dilution. This
30 makes it possible to use an induced Fluorescence
Resonance Energy Transfer (Genome Research, 2002,
12:1401-1407) interaction between SYBR Green I dye and
the ROX label of the probe to generate a fluorescence
signal that is related to the existence of double-strand

DNA entailing probe and amplified target sequences. To create the melt-curves, the membrane was placed between glass plates under blue light (470nm peak wavelength, suitable for SYBR Green I dye excitation), and its

5 temperature was increased from 30°C to 85°C via a custom-made heating device at a rate of 3°C per minute whilst the fluorescence emitted by each array feature at 630nm (ROX label emission) was measured twice per second via CCD camera imaging. Custom software was then used to

10 quantify the camera images and construct graphic plots of the resulting denaturation melt curves.

Results

The melt-curve graphical plots created for the assayed

15 membrane array features (each feature representing the result for one individual) were a) raw data plots of fluorescence versus temperature (Figure 1), and b) plots of fluorescence versus temperature normalized to an equivalent starting fluorescence value (Figure 2).

20 Additionally, plots were generated to display the negative derivative of fluorescence with respect to temperature versus temperature, and this was done for both the raw and the normalized primary data curves (giving Figure 3 and Figure 4 respectively). In all these

25 plots, the 12 tested DNAs could be seen to fall into one of three distinct clusters; i) curves with a maximal denaturation rate at a relatively low temperature (point A), ii) curves with a maximal denaturation rate at a relatively high temperature (point B), and iii) curves

30 with maximal denaturation rates at both low and high temperatures. Higher melting temperatures correspond to perfectly matched (more stable) probe-target duplexes, whilst lower melting temperatures correspond to mismatched (less stable) probe-target duplexes, so the

three observed curve types equated respectively to i)
homozygotes mismatched to the utilized probe sequence
(that is, matching the alternate 'A' nucleotide allele),
ii) homozygotes matched to the utilized probe sequence

5 ('C' nucleotide allele), and iii) heterozygotes (carrying
both the 'C' and the 'A' nucleotide alleles). Grouping
lines were drawn manually upon each of the graphical
plots (see Figures 1-4) to group the melt curves into the
apparent clusters, and these clusters were then assigned

10 to the three genotype categories based upon the
interpretation principles described above. Four
individuals of each genotype category were thus
identified, and these sample assignments were consistent
across all of the graphical plots considered. Additional

15 samples may be subsequently examined by the same
genotyping method using these established grouping lines
for marker rs1133104 to automatically establish the
genotype class for any further samples subjected to the
same analysis.

20

3. A method according to claim 1 or claim 2 wherein said grouping lines are applied to said population of melt curves by a user.

5 4. A method according to claim 3 wherein the user applies the grouping lines to a displayed image of said population of melt curves using a graphic interface.

5. A method according to claim 1 or claim 2 wherein
10 said grouping lines are applied to said population of melt curves by a data processor.

6. A method according to claim 5 wherein said grouping lines are applied by

15 (i) tracking the Y-value distribution of said melt curves along the X-axis,

(ii) identifying one or more regions in which said melt curves separate into distinct clusters; and,

(iii) applying one or more grouping lines to define each
20 said cluster.

7. A method according to claim 5 wherein said grouping lines are applied by

(i) applying a plurality of candidate lines to said
25 population of melt curves, and;
(ii)
identifying one or more candidate lines which only intersect a discrete cluster of curves within said population as grouping lines.
30

8. A method according to any one claims 1 to 7 comprising;

applying a plurality of grouping lines to the population of melt curves,

identifying one or more grouping lines intersected by the melt curve of the sample, and

5 applying an assignment algorithm to determine the genotype category of the nucleic acid sample.

9. A method according to claim 8 comprising;

assigning an order of precedence to the one or more

10 grouping lines, and;

assigning the nucleic acid sample to the genotype category of the grouping line with the highest precedence.

15 10. A method according to any one claims 1 to 9 wherein the genotype category is selected from homozygous for sequences matched with an allelic reference sequence, homozygous for sequences mismatched with an allelic reference sequence, or heterozygous.

20

11. A method according to any one of claims 1 to 10 wherein said melt curves plot changes in the output signal relative to the hybridization conditions.

25 12. A method according to any one of claims 1 to 10 wherein said melt curves plot the positive or negative first derivative of changes in the output signal relative to the hybridization conditions.

30 13. A method according to any one of the preceding claims comprising normalising said population of melt curves prior to applying said grouping lines.

14. A computer program product carrying computer-readable code for performing the method of any one of claims 1 to 13.

5   15. Computer-readable code for performing the method of any one of claims 1 to 13.

16. A computer system configured to perform the method of any one of claims 1 to 13.

10

17. A DNA hybridization device having an output signal detector and a computer system according to claim 16 for analyzing data obtained by the detector.
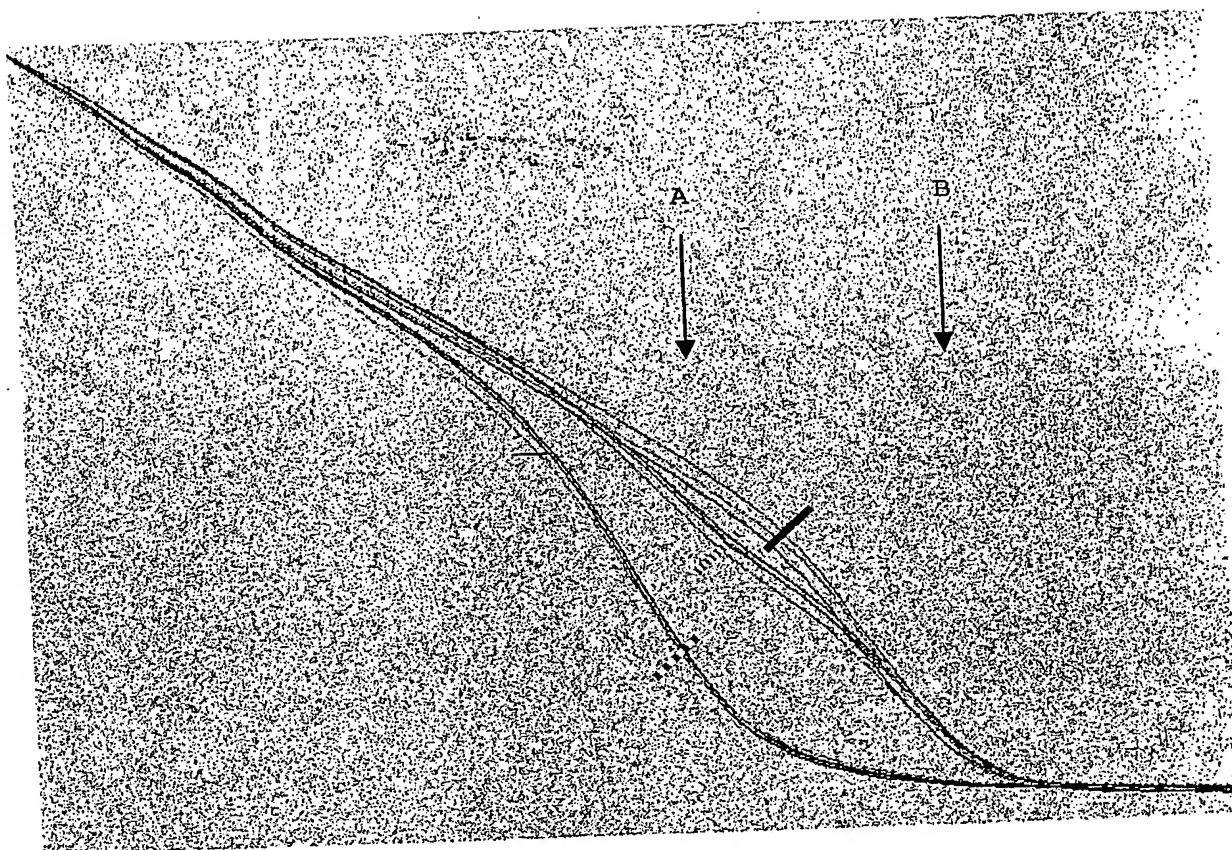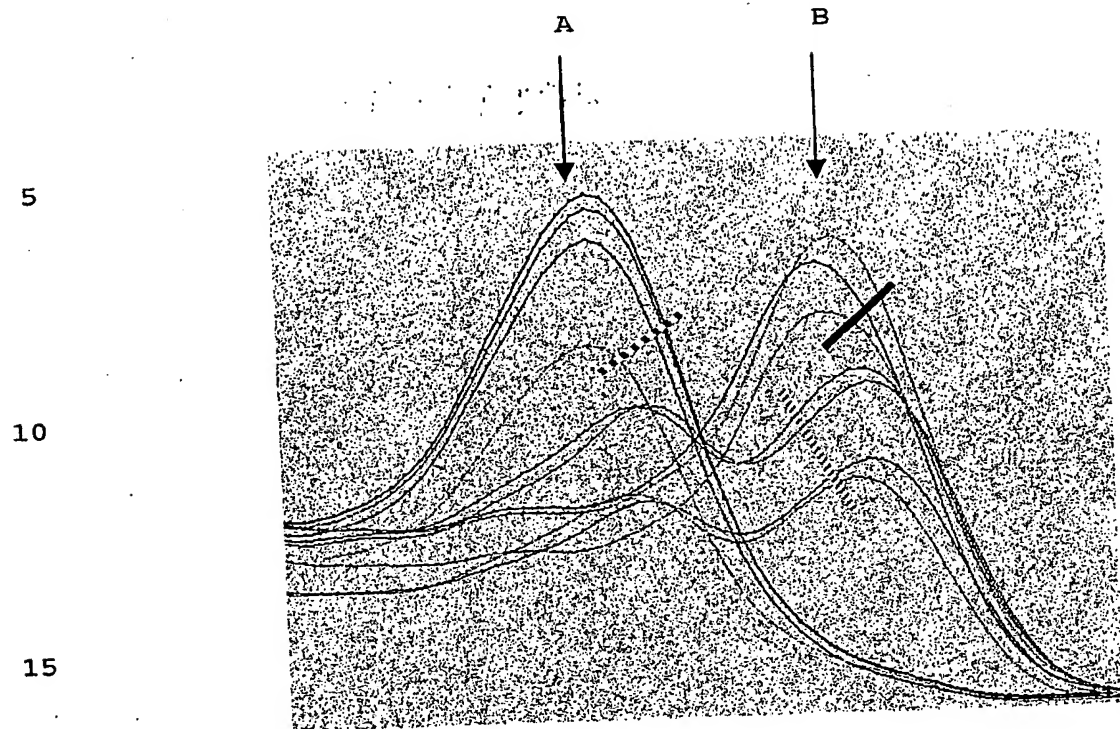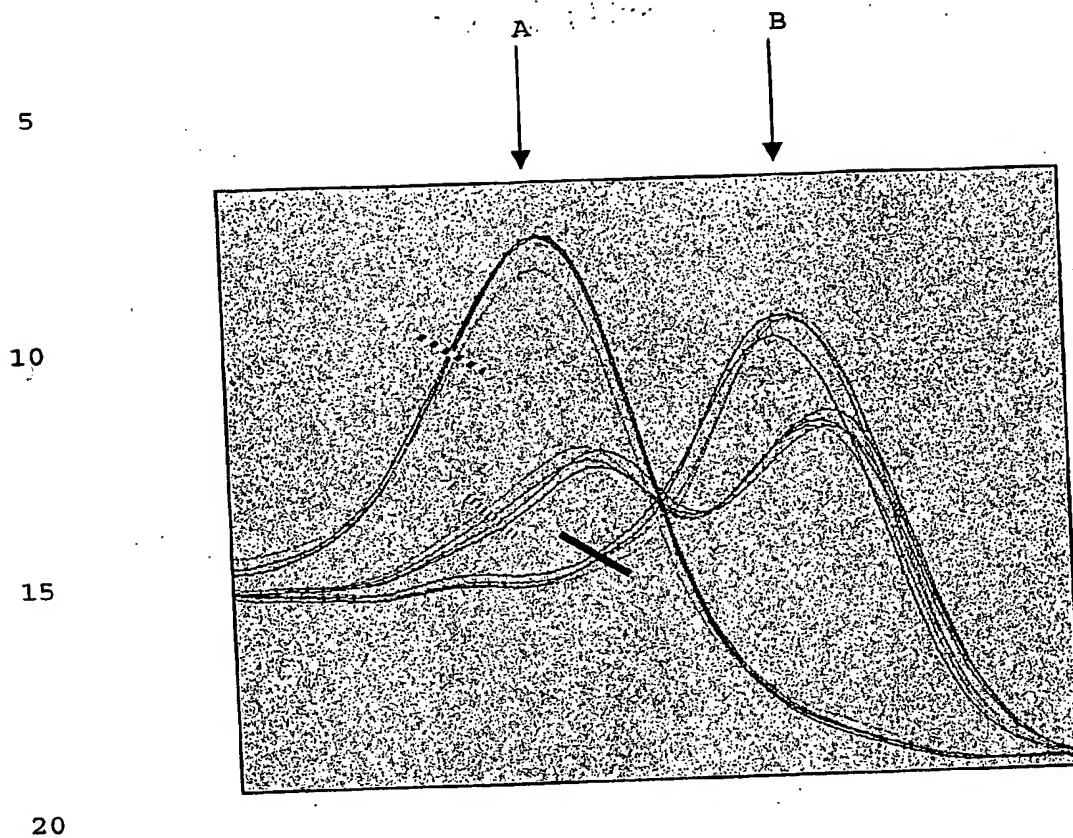
Figure 2

Figure 3

Figure 4